General

Guideline Title

Guidelines on the use of multicolour flow cytometry in the diagnosis of haematological neoplasms.

Bibliographic Source(s)

Johansson U, Bloxham D, Couzens S, Jesson J, Morilla R, Erber W, Macey M, British Committee for Standards in Haematology. Guidelines on the use of multicolour flow cytometry in the diagnosis of haematological neoplasms. Br J Haematol. 2014 May;165(4):455-88. [130 references] PubMed

Guideline Status

This is the current release of the guideline.

Recommendations

Major Recommendations

Definitions for the quality of evidence (A–C) and strength of recommendations (Strong [Grade 1], Weak [Grade 2]) are given at the end of the "Major Recommendations" field.

Instrumentation

1. In-house evaluation of multicolour flow cytometry (MFC) cytometer prior to purchasing (1B).
2. Choose lasers with known good stability and filters suitable for use with available reagents (1C).
3. Photomultiplier tube (PMT) voltages should be optimized and set to maximize signal-noise ratio and to allow for measuring all antigen expression within the cytometer's linear range (1A).
4. Changes in window extension time must include a check of light scatter threshold settings (1A).
5. It is preferable to have one, single cytometer configuration for all clinical work (2A).
6. It is strongly advised to achieve a stable cytometer performance through applying the following procedures:
    a. Daily system performance quality control (QC) that is document controlled (1A).
    b. Tracking PMT voltages to achieve standard mean fluorescence intensities (MFIs), correcting for daily drift and laser changes. This should preferably be done using specialized software (1A).
7. Acquire light scatter parameters to allow for coincidence monitoring (1A).
8. Use logarithmic forward scatter (FSC) and side scatter (SSC) for analysis of red cells and platelets (1A).
9. View fluorescence on a logarithmic scale (except for DNA analysis) and use logicle displays.
10. Use software to set compensation and, if required, the median fluorescence method to investigate if compensation is correct (1A).
11. Ensure compensation matrices are appropriately applied to the correct experiments, and kept up-to-date with arrival of new lots; this process should be document controlled (1C).

Multicolour Tube Design and Validation

1. Selection of antigens and other reagents to include in the panel:
    a. For routine diagnostic and prognostic investigations, antigens investigated should be based on known, published evidence (1C).
    b. Reagent specificity should be known or checked and documented (1C).
    c. The type of sample investigated should be taken into account (1A).
2. Validation of reagent combinations:
    a. Optimize antigen/fluorochrome combinations. Select fluorochrome to match antigen expression intensity and, at the same time, consider resolution sensitivity in channels affected by the spill-over (1A).
    b. Run fluorescence minus one (FMO) controls for all new combinations, to check for artefacts and compromised assay sensitivity, and for learning expression patterns (1A).
    c. Check for steric hindrance (1A).
3. All analysts and interpreters to have a very good knowledge of expression patterns, including FMOs, for all multicolour combinations used (1A).

Reagent Handling: Antibody Cocktails

1. Avoid reagents not suitable for use in cocktails (1C).
2. Good reagent house-keeping:
    a. All reagents should be tested for lot-to-lot variation in immunoglobulin/dye concentration, signal strength and spectral spill-over characteristics (1C).
    b. Titrate concentration and re-compensate as required (1C).
3. Validate cocktail shelf life (1C). Suggested validation procedures include:
    a. Label antibody-capture beads or lyophilized cells over time: once a day for as long as it is anticipated the cocktail will need to last.
    b. Compare performance of cocktail with that of singly aliquoted antibodies on fresh specimens. As above, repeat this test on a daily basis (with a new, fresh specimen) and for as long as it is anticipated the cocktail will be in use.
4. Record the lot number of each individual antibody, date, and name of the person preparing the cocktail (see Figure 4 in the original guideline document) (1A).
5. Use fresh vials every time; ideally made of dark glass or polypropylene (1A).
6. Aliquot antibodies using the same pipette to avoid volume errors (1A).
7. Use the same relative reagent volumes as if using the reagents individually. The correct volume of cocktail to be used in an individual test will be the sum of that required for each antibody if it was being used singularly e.g., 8 x 10 µl (1A).
8. Validate each new cocktail lot to ensure it contains the correct antibodies (1A): Comparison of singly aliquoted antibodies with the new cocktail is not required if the cocktail expression patterns are well known to the analyst. Normal peripheral blood (PB) or bone marrow (BM) samples could be used and results recorded.
9. Keep a complete audit trail for each cocktail used for any patient (1A).

Pre-Analytical Variables: Specimen

1. Sample age and quality must be assessed prior to analysis (1A).
2. Assessment of morphology should be done in immediate conjunction with flow analysis, to assist the selection of a diagnostic pathway and to assess sample quality (2A).
3. Ethylenediaminetetraacetic acid (EDTA) is recommended as a universal anticoagulant as it also permits morphological assessment (2B).
4. Use live/dead stain on tissue sample preparations (2A).
5. Sample storage (1B):
    a. Cerebrospinal fluid: Culture media or Transfix for transport media; storage at +4°C, for maximum 24–48 h, or 72 h for Transfix. Note that morphological evaluation is not possible if Transfix is used. If transport media is not used: immediate analysis.
    b. Other fluids: Storage at +4°C, for maximum 24 h. Use of transport media has not been validated and may be difficult to introduce into clinical practice.
    c. Tissue preparations from fresh specimens: Tissue culture media for transport and +4°C storage for maximum 24–48 h. Where transport media is not used, store in saline or similar at +4°C and analyse within 24 h.
    d. PB/BM:
        i. Lymphoproliferative disease investigations: General, at room temperature for up to 48 h; however, for basic B-cell clonality screen – storage at +4°C for up to 72 h.
        ii. Mono-myeloid investigations, including validation of normal patterns: should be kept at a similar temperature and analysed within 48 h.

iii. High-grade lymphomas: Immediate analysis is recommended.

Pre-Analytical Variables: Labelling

1. Processing:
    a. Health and Safety considerations: Risk assessments should be carried out. Fixation should be considered for tissue analysis. If fixation is used, it is strongly recommended that previously untested antibodies and/or conjugates, particularly tandem fluorochromes, be validated prior to use (1A).
    b. Antibody volume must be titrated by each laboratory to ensure that optimum final concentrations of antibodies are used to achieve maximum separation between positive and negative populations thereby minimizing background binding (1A).
    c. Red cell lysis is recommended over density centrifugation. Home-made lysing agents need validating, including a pH test, prior to use. Lysis after labelling is recommended (1A).
        i. Immunoglobulin stain requires pre-wash and/or prelyse (1A).
    d. Wash after labelling and lysis is recommended to reduce non-specific fluorescence. For paroxysmal nocturnal haemoglobiuria red blood cell analysis, two washes are recommended (1B).
2. Indications for use of controls (1B):
    a. Suspected artefactual results: FMO test.
    b. No internal control present: FMO test, isotype control.
    c. Intracellular labelling, to monitor the effects of sample preparation on autofluorescence and non-specific binding: Isotype control, unlabelled processed sample, and/or FMO.
    d. Monitor autofluorescence: acquire unlabelled processed sample.
3. Isotype or isoclonic controls should not be used for reporting percentage positive cells (1A).

Data Acquisition

1. All data files should have clear, sample-specific labelling and identification that prevents mix-up possibilities during data analysis, particularly where data migration to other software is used (1A).
2. An appropriate number of total events must be acquired, particularly for high sensitivity investigations (0.01% and below) (1C).
3. Fluidics and coincidence should be monitored during acquisition (1A).
4. Document control should include compensation matrices used (1C).

Data Analysis

1. All data should be scrutinized for quality prior to diagnostic analysis. Checks should include:
    a. Sample quality – overall light scatter and fluorescence patterns (1A).
    b. Cytometer QC: Fluidics and coincidence (1A).
    c. Labelling process: Internal controls (1B).
2. Gating strategy should take light scatter, CD45 and relevant lineage antigens into account (1B).
3. Logicle view should be used wherever possible (1A).
4. Consider doublet exclusion for populations that contain aggregates and some dividing cells, and for high resolution/minimal residual disease (MRD) analysis (1B).
5. Any reported abnormal populations, particularly high resolution and MRD results, should be "vetted" by back-gating in all light scatter and fluorescence channels (1B).

Reporting

1. *Sample information*: Referral centre's specimen number, date taken, date received, date processed, age and overall quality of sample at time of analysis. For BM samples this should include whether it is aparticulate, haemodilute or partially clotted (see 'Limitations and disclaimers' below). The type of sample and tests requested should be clearly identified. Relevant details from a full blood count may be included where this would be helpful; for example, either within the flow report for lymphocytosis queries, or as part of the integral report for acute leukaemia or myelodysplastic syndrome (MDS) (1A).
2. *Diagnostic context*: The flow report should form part of a National Institute for Health and Care Excellence (NICE) improving outcomes guidance (IOG)-compliant integrated report (1A). However, a diagnosis should not be delayed by this process and it is recommended that flow reports are authorized as soon as possible. If a flow report is sent out without an accompanying morphology report, statements such as 'correlate with local sample for morphology' or 'morphology to follow' should be included. Points a-c below would be part of the integrated report. They are mentioned here as certain queries may result in an interim, free-standing flow report.
    a. *Centre information and contact details*: Clinical Pathology Accreditation or similar laboratory accreditation body, laboratory

address, telephone, fax number and an appropriate contact name, normally clinical and/or scientific lead.

b. *Patient's demographic identification*: Patient's surname, forename, date of birth, gender, hospital number and National Health Service number (1A).

c. *Information from the referring institution*: Name, address, telephone and fax numbers of the referring doctor. Referring centre should also provide a freshly made film for PB and BM specimens and relevant clinical details. The final report should mention if this was not the case (1A).

3. *Flow cytometry (FC) data*:

a. PB reports for general lymphocytosis queries should include a breakdown of lymphoid populations: T cells, B cells, natural killer (NK) cells, and large granular lymphocytes (LGL), as percentage of lymphocytes or absolute counts. The absolute count of the cells of interest should be stated, where this affects or assists the diagnosis, e.g., monoclonal B-cell lymphocytosis (MBL)/small lymphocytic leukaemia (SLL)/chronic lymphocytic leukaemia (CLL) and NK/LGL populations (Swerdlow et al., 2008) (1A).

b. Presence of abnormal cells in BM should be expressed as a percentage of either nucleated cells or $CD45^+$ cells. It should be clear which parameter was used. If $CD45^+$ events are used as reporting parameter, then CD45-negative white blood cells (WBC), for example certain plasma cells or malignant precursor B cells, need to be added to the CD45 count to reach the total number of WBC (1A).

c. For BM samples, a summary of all major populations as % of total cells, so called flow differentials, can be useful, for example, for MDS analysis. However the sample quality must be considered. The flow sample may or may not be representative (see point 'Limitations and disclaimers' below). A flow differential may also be helpful to illustrate an unrepresentative sample. This is particularly important for uniquely flow-based prognostic investigations and for trouble-shooting discrepant results. In addition, note that levels of BM nucleated red blood cells (RBCs) are normally reduced after sample processing for FC (1A).

d. Description of abnormal population: This should include a text summary of the phenotype and light scatter characteristics. Normal/abnormal antigen expression intensities (for example weak/moderate/strong) and maturation/expression patterns should be indicated in the context of the specimen analysed. Include additional comments on proliferation and/or prognostic markers (1A).

e. Listing/table of percentages and/or reporting of isolated antigens not relevant for the workup are strongly discouraged (1A).

f. For rare event analysis, where the population size is reported at 0.05% or below: state the number of rare events and total events acquired (2C). For well-established MRD assays where assay sensitivity is known, this may be stated, provided a sufficient number of events were acquired.

g. Use CD nomenclature. Where antigen expression may be intracellular (cytoplasmic or nuclear) as well as surface, it is important to define the description with the prefix c, cyt, for cytomplasmic, n for nuclear, or ic, for intracellular (2A).

h. A statement of major antigens/panels investigated is recommended particularly for reports concluding 'no abnormalities seen'. This can be kept brief and relevant to the query. For example, a B-non-Hodgkin lymphoma staging marrow report may read '$CD19^+$ B cells present at 5% of $CD45^+$ cells in this sample. These have normal CD19, CD20, CD5, CD10, CD45 and light chain expression patterns' or 'B-cell screen applied, no abnormal B cells detected' (1B).

4. *Limitations and disclaimers*: Any issues about sample quality should be described (2A). References to known FC pitfalls relevant to the report should be included.
5. *Summary/Conclusion*: a summary of the findings, differential diagnostic conclusions and/or comments (2A). Recommendation of additional relevant tests to establish or support a diagnosis, such as histology, cytogenetic analysis and/or molecular tests is desirable (2A).
6. *Reporter/Authorizer*: FC reports must be interpreted and authorized by a named accredited scientist or consultant with specialist training in MFC for leukaemia and lymphoma immunophenotyping and with the appropriate haematology oncology diagnostics training and experience (1A).
7. The inclusion of multicolour plots can help the understanding of results, gating strategy and the proportion of cells in the specimen received for FC (2A).
8. For new diagnoses, the availability of MRD targets could be stated (2A).

Training

1. Clearly defined roles and levels of responsibilities for all staff are essential and training should be tailor-made to suit staff at the level of their responsibility (1A).
2. Review and record individual competencies on an annual basis (2A).
3. Senior staff new to MFC should train in an experienced MFC laboratory and attend external courses (1A).
4. Exposure to 100 new clinical cases/annum is a minimum for all staff who interpret MFC data and reports results (1C).

Validation Procedures and Topics for Audit

Standard operating procedures for all areas of the laboratory require validation. These and all controlled documents may be audited; in particular:

1. Cytometer:
    a. Documentation of cytometer optimization and daily cytometer QC that monitors and logs cytometer setup parameters. It is strongly recommended that this include laser delay checks and adjustment if required. Laboratories should have a fixed tolerance for the drift allowed in measured fluorescence: a manual or automated tracking system, resulting in steady MFI values, should be document controlled (1A).
    b. Cytometer hardware maintenance and error log, particularly laser replacement/realignment (1A).
2. Reagents:
    a. Records for titration and fluorescence spill-over monitoring (1A).
    b. Records of compensation matrices used for analysis (1A).
    c. Documentation of reagent and home-made reagent cocktails that enable clear identification of lots used for any given sample allowing a complete audit trail to be achieved (1A).
3. Panel validation procedures: Records of validation process for MFC panels used, and any changes made to them. If published evidence is used, this should be accessible (1A).
4. Training: Individual staff records documenting specialized MFC training and competency. This should include standard continued professional development aspects, such as internal and external training courses and participation in quality assurance programmes (1A). In addition, past and current case load should be monitored. It should be ensured that this includes a cross section of haematological neoplasms.

Definitions:

Quality of Evidence

The quality of evidence is graded as high (A), moderate (B) or low (C). To put this in context, it is useful to consider the uncertainty of knowledge and whether further research could change what is known or is certain.

(A) High: Further research is very unlikely to change confidence in the estimate of effect. Current evidence derived from randomized clinical trials without important limitations.

(B) Moderate: Further research may well have an important impact on confidence in the estimate of effect and may change the estimate. Current evidence derived from randomized clinical trials with important limitations (e.g., inconsistent results, imprecision - wide confidence intervals or methodological flaws – e.g., lack of blinding, large losses to follow up, failure to adhere to intention to treat analysis), or very strong evidence from observational studies or case series (e.g., large or very large and consistent estimates of the magnitude of a treatment effect or demonstration of a dose-response gradient).

(C) Low: Further research is likely to have an important impact on confidence in the estimate of effect and is likely to change the estimate. Current evidence from observational studies, case series or just opinion.

Strength of Recommendations

Strong (Grade 1): Strong recommendations (Grade 1) are made when there is confidence that the benefits do or do not outweigh harm and burden. Grade 1 recommendations can be applied uniformly to most patients. Regard as "recommend".

Weak (Grade 2): Where the magnitude of benefit or not is less certain a weaker Grade 2 recommendation is made. Grade 2 recommendations require judicious application to individual patients. Regard as "suggest".

Clinical Algorithm(s)

None provided

Scope

Disease/Condition(s)

Haematological neoplasms (leukaemia and lymphoma)

Guideline Category

Diagnosis

Clinical Specialty

Hematology

Oncology

Intended Users

Clinical Laboratory Personnel

Physicians

Guideline Objective(s)

To provide healthcare professionals with clear guidance on the use of multicolour (or polychromatic) flow cytometry (MFC) for leukaemia and lymphoma immunophenotyping of peripheral blood (PB), bone marrow (BM), body fluids and tissue specimens

Target Population

Patients with suspected haematological neoplasms (leukaemia and lymphoma)

Interventions and Practices Considered

1. Consideration of types and settings of instrumentation
2. Multicolour tube design and validation (selection of antigens/reagents and validation of reagent combinations)
3. Parameters for reagent handling: antibody cocktails
4. Consideration of pre-analytical variables: specimen and labeling protocols
5. Data acquisition and analysis
6. Reporting procedures
7. Training of staff and defining roles
8. Validation and audit of procedures

Major Outcomes Considered

- Diagnostic accuracy of tests
- Sensitivity and specificity of tests

Methodology

Methods Used to Collect/Select the Evidence

Searches of Electronic Databases

Description of Methods Used to Collect/Select the Evidence

The production of these guidelines involved literature review from 2009 to 2014 using PubMed and Google Scholar. No exclusion criteria were used. Inclusion criteria varied widely; the guidelines encompass several subject areas, and inclusion criteria were specific and related to the subject dealt with for each section of the guidelines. Search terms used in literature searches may be found as part of the headings. Generally, 'flow cytometry' would be included. However, for example, in the section on anticoagulants, search terms would include 'anticoagulant', 'peripheral blood' (or 'bone marrow'/CSF/other types of samples) and 'antigen' or 'temperature' or other terms, sometimes very specific, such as 'monocyte, CD13, anticoagulant'.

Number of Source Documents

Not stated

Methods Used to Assess the Quality and Strength of the Evidence

Weighting According to a Rating Scheme (Scheme Given)

Rating Scheme for the Strength of the Evidence

Quality of Evidence

The quality of evidence is graded as high (A), moderate (B) or low (C). To put this in context, it is useful to consider the uncertainty of knowledge and whether further research could change what is known or is certain.

(A) High: Further research is very unlikely to change confidence in the estimate of effect. Current evidence derived from randomized clinical trials without important limitations.

(B) Moderate: Further research may well have an important impact on confidence in the estimate of effect and may change the estimate. Current evidence derived from randomized clinical trials with important limitations (e.g., inconsistent results, imprecision - wide confidence intervals or methodological flaws – e.g., lack of blinding, large losses to follow up, failure to adhere to intention to treat analysis), or very strong evidence from observational studies or case series (e.g., large or very large and consistent estimates of the magnitude of a treatment effect or demonstration of a dose-response gradient).

(C) Low: Further research is likely to have an important impact on confidence in the estimate of effect and is likely to change the estimate. Current evidence from observational studies, case series or just opinion.

Methods Used to Analyze the Evidence

Systematic Review

Description of the Methods Used to Analyze the Evidence

These guidelines have been prepared using the GRADE (Grading of Recommendations Assessment, Development and Evaluation) nomenclature for assessing the quality of evidence (see the "Rating Scheme for the Strength of the Evidence" field).

Methods Used to Formulate the Recommendations

Expert Consensus

Description of Methods Used to Formulate the Recommendations

The guideline group comprised representatives of UK-based clinical flow cytometry (FC) experts and clinical representatives. The writing group produced the draft guidelines, which were subsequently revised by consensus by members of the Haemato-oncology Task Force of the British Committee for Standards in Haematology (BCSH).

These guidelines have been prepared using the GRADE (Grading of Recommendations Assessment, Development and Evaluation) nomenclature for providing strength of recommendations (see the "Rating Scheme for the Strength of the Recommendations" field).

Rating Scheme for the Strength of the Recommendations

Strength of Recommendations

Strong (Grade 1): Strong recommendations (Grade 1) are made when there is confidence that the benefits do or do not outweigh harm and burden. Grade 1 recommendations can be applied uniformly to most patients. Regard as "recommend".

Weak (Grade 2): Where the magnitude of benefit or not is less certain a weaker Grade 2 recommendation is made. Grade 2 recommendations require judicious application to individual patients. Regard as "suggest".

Cost Analysis

A formal cost analysis was not performed and published cost analyses were not reviewed.

Method of Guideline Validation

External Peer Review

Internal Peer Review

Description of Method of Guideline Validation

The guidelines were then reviewed by a sounding board of approximately 50 United Kingdom (UK) Haematologists and flow cytometry (FC) experts, the British Committee for Standards in Haematology (BCSH) and the British Society for Haematology (BSH) Committee, and comments incorporated where appropriate.

Evidence Supporting the Recommendations

References Supporting the Recommendations

Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW, editor(s). WHO classification of tumors of haematopoietic and lymphoid tissues. Geneva (Switzerland): WHO Press; 2008.

Type of Evidence Supporting the Recommendations

The type of supporting evidence is identified and graded for most recommendations (see the "Major Recommendations" field).

Benefits/Harms of Implementing the Guideline Recommendations

Potential Benefits

Appropriate use of multicolour flow cytometry in the diagnosis of haematological neoplasms to improve the accuracy and reproducibility of results

Potential Harms

- Generally, samples older than recommended may be selected for analysis; this decision should be made by senior, experienced staff and must take into account any risks of misleading information, for example, a negative result from a cerebrospinal fluid (CSF) that is too old for analysis.
- Washing after labelling is recommended to reduce nonspecific fluorescence. Exceptions to this include enumeration techniques where bead counting technology is used. For example, CD4 or CD34 enumeration and nucleated red blood cell (nRBC) evaluation all follow a 'lyse-no-wash' protocol. An increased number of washes increase the risk of losing cells, particularly fragile cells such as plasma cells. Normally, one wash after antibody labelling is sufficient.

Qualifying Statements

Qualifying Statements

While the advice and information in these guidelines is believed to be true and accurate at the time of going to press, neither the authors, the British Society for Haematology, nor the publishers accept any legal responsibility for the content of these guidelines.

Implementation of the Guideline

Description of Implementation Strategy

An implementation strategy was not provided.

Implementation Tools

Resources

For information about availability, see the *Availability of Companion Documents* and *Patient Resources* fields below.

Institute of Medicine (IOM) National Healthcare Quality Report Categories

IOM Care Need

Living with Illness

IOM Domain

Effectiveness

Identifying Information and Availability

Bibliographic Source(s)

Johansson U, Bloxham D, Couzens S, Jesson J, Morilla R, Erber W, Macey M, British Committee for Standards in Haematology. Guidelines

on the use of multicolour flow cytometry in the diagnosis of haematological neoplasms. Br J Haematol. 2014 May;165(4):455-88. [130 references] PubMed

Adaptation

Not applicable: The guideline was not adapted from another source.

Date Released

2014 May

Guideline Developer(s)

British Society for Haematology Guidelines - Professional Association

Source(s) of Funding

British Committee for Standards in Haematology

Guideline Committee

British Committee for Standards in Haematology (BCSH) Writing Group

Composition of Group That Authored the Guideline

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Financial Disclosures/Conflicts of Interest

None of the authors have declared a conflict of interest.

Guideline Status

This is the current release of the guideline.

Guideline Availability

Electronic copies: Available from the British Journal of Haematology Web site .

Print copies: Available from the British Committee for Standards in Haematology; Email: bcsh@b-s-h.org.uk.

Availability of Companion Documents

The appendices of the original guideline document contain information on the following:

- Suggested multicolour flow cytometry (MFC) competency levels and training resources
- Controls (fluorescence minus one [FMO] controls, isotype controls, internal controls, isoclonic controls, process controls)
- Anticoagulants
- Published panels and gating strategies
- Examples of MFC reports

Patient Resources

None available

NGC Status

This NGC summary was completed by ECRI Institute on July 16, 2014.

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